Redistribution of cell death-inducing DNA fragmentation factor-like effector-a (CIDEa) from mitochondria to nucleus is associated with apoptosis in HeLa cells

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Abstract. Cell death-inducing DFF[DNA fragmentation factor]-like effector-a (CIDEa), may initiate apoptosis by disrupting a complex consisting of 40-kDa caspase-3-activated nuclease (DFF40/CAD) and its 45-kDa inhibitor (DFF45/ICAD). CIDEa, however, was found to be localized in mitochondria. We have performed immunodetection of CIDEa in whole cells and subcellular fractions of HeLa cells adapted for a tetracycline-inducible CIDEa expression. Using immunocytochemistry we observed redistribution, enhanced upon treatment with camptothecin or valinomycin, of CIDEa to nucleus. Similarly, CIDEa content increased in the nuclear fraction but decreased in cytosolic fraction in cells treated to initiate apoptosis. We hypothesize that CIDEa is sequestered in mitochondria while transfer of this potentially dangerous protein from mitochondria into nucleus intensifies or even initiates apoptosis.

Key words: CIDEa — Mitochondria — Apoptosis — Migration to nucleus — HeLa cells

Abbreviations: BSA, bovine serum albumin; CAD, caspase-activated DNase; CCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone; DFF, DNA fragmentation factor; CIDEa, cell death-inducing DFFa-like effector; DTT, dithiothreitol; EDTA, ethylenediamintetraacetic acid; ICAD, inhibitor of CAD; PBS, phosphate buffered saline; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; TUNEL, TdT-mediated dUTP nick end labeling.

Introduction

Besides their function as metabolic powerhouses, mitochondria are one of major "decision makers" in apoptosis initiation and as gatekeepers of other ways of cell death (Cheng et al. 2006; Kim et al. 2006). Relay of information signaling that involves mitochondria is mediated by proteins possessing the ability to migrate between mitochondria and cytosol or other cell organelles or compartments, notably to the nucleus where subsequent signaling cascades may be initiated. On one hand, a "regular" protein import proceeds into mitochondrion (import of nuclear-coded mitochondrial proteins) bringing in either executive or regulatory proteins (Baker et al. 2007). Among the latter, PKC-, tyrosine- and other kinases were found to access at least the intermembrane space (Salvi et al. 2005). On the other hand, an export of signaling proteins into the cytosol or other cell organelles exists and plays an essential role in life and death (Cheng et al. 2006; Garrido et al. 2006; Kim et al. 2006). Thus, protein export mechanisms for apoptosis mediators such as cytochrome c, Smac/Diablo, apoptosis-inducing factor or EndoG serve as well established, but not completely understood examples (Cheng et al. 2006; Kim et al. 2006; Varecha et al. 2007).

Bearing in mind the above principles, it is not surprising that proteins called cell death-inducing DFFa (DNA fragmentation factor a)-like effector-a,b,3 (CIDEa, CIDEb, CIDE3/FSP27) have been found in mitochondria (Inohara et al. 1998). The three CIDE proteins have different tissue distributions for their mRNA and their physiological roles specific to given tissues are unknown. CIDE proteins display high homology to the N-terminal sequence of both subunits of the

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heterodimeric DFF, which consists of the 40-kDa caspase-3-activated nuclease (DFF40/CAD), and its 45-kDa inhibitor (DFF45/ICAD). The DFF45/DFF40 complex is cleaved by caspase-3 during apoptosis and thereafter free DFF40 causes apoptotic DNA fragmentation and chromatin condensation (Inohara et al. 1999; Bayascas et al. 2004). CIDE-induced apoptosis is not sensitive to caspase inhibitors but is inhibited by DFF45 (Inohara et al. 1998). The N-domain of CIDE proteins most probably binds to the homologous domain of DFF45 opposing the factor's inhibitory as well as chaperone effect on DFF40 (Lugovskoy et al. 1999; Neimanis et al. 2007). The nuclease is then able to cleave DNA. It is not yet fully established, whether the DFF45/DFF40 complex is formed in the nucleus or in the cytosol. However, nuclear import studies suggested that the DFF45/DFF40 complex is imported more readily than its components (Neimanis et al. 2007), thus preferring the model of cytosolic formation of the complex. If this was exclusive modus operandi of DFF, then CIDEa must migrate to nucleus in order to bind DFF45 and induce apoptosis. If sole DFF40 nuclease could migrate into the nucleus, apoptosis might be induced by CIDE-mediated dissociation of the DFF45/DFF40 complex in the cytosol.

The situation is more complex, because several studies localized CIDEa (Zhou et al. 2003), CIDEb (Chen et al. 2000) and CIDE3 (Liang et al. 2003) in mitochondria. A consensus has been found that while the N-CIDE domain located within the N-terminal is required for binding to DFF45 or DFF40 (Inohara et al. 1998; Lugovskoy et al. 1999; Chen et al. 2000), the C-CIDE domain within the C-terminal is required for mitochondrial localization and apoptosis (Inohara et al. 1998; Chen et al. 2000). The latter finding contrasts with the obligatory N-terminal localization of mitochondrial addressing sequences (presequences or scattered motifs). Employing dedicated software, e.g. *http://psort.ims.u-tokyo.ac.jp*, we were unable to locate a mitochondrial addressing sequence within either CIDEa or CIDEb amino acid sequences. We may assume that docking in mitochondria is just ensured by the C-CIDE domain. Migration of tagged CIDEb, for example, from mitochondria to nucleus (or to cytosol) was missing in case of apoptosis induced by staurosporine and etoposide (Chen et al. 2000). Because the two drugs affect primarily Bak/Bax-dependent apoptosis, we selected other effectors, including camptothecin and valinomycin, and endeavored to investigate the issue of CIDEa migration.

Materials and Methods

Inducible CIDEa expression

An open reading frame clone of human CIDEa in an entry vector pENTR221 (Invitrogen) has been transposed using a clonase reaction into a vector T-REx pDEST30 (Invitrogen) for a tetracycline-induced expression, obtaining pDEST30-CIDEa plasmid containing the full open reading frame for CIDEa inserted downstream from *tet* repressor binding site. The HeLa cell line overexpressing the tetracycline repressor (Invitrogen) was cultured in the presence of antibiotic Blasticidin (1 μg/ml) at 37°C in a humidified incubator with 5% CO_2 in the Eagle's minimum essential medium supplemented with 2 mmol/l L-glutamine, 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Transfections were performed using 1 μl Lipofectamine 2000™ (Roche) per 1 μg DNA with estimated efficiency up to 50%. Following transfection cells were allowed to stabilize for 24 h and then subjected to further treatments.

Initiation of apoptosis

The pDEST30-CIDEa-transfected T-REx-HeLa cells were subjected to tetracycline (1 μg/ml) for defined time intervals, thus obtaining a time-course of induced CIDEa expression. In experiments where apoptosis initiators were used, cells were subjected to tetracycline treatment for 8 h followed by 2 h incubations with following apoptosis initiators: a DNA-topoisomerase I complex inhibitor camptothecin (2 μmol/l); a protonophore CCCP (2 μmol/l); a potassium ionophore valinomycin (2 μmol/l); and a calcium ionophore A23187 (2 μmol/l). Extent of apoptosis was assayed using caspase-3 activity, TUNEL assay and cell death assay assessing cell morphology.

Preparation of subcellular fractions

Minor modifications of previously described method (Carcamo et al. 2002) have been adapted. T-REx-HeLa cells were washed twice with 2 ml of ice-cold PBS and scraped into 1 ml of PBS. Suspension was centrifuged (1500 × *g*/5 min/4°C), the pellet was resuspended by gentle pipetting in 300 μl of ice-cold buffer A (10 mmol/l HEPES, pH 7.9; 10 mmol/l KCl; 1.5 mmol/l $MgCl_2$; 0.5 mmol/l DTT; 0.1% (v/v) NP-40), incubated for 10 min on ice, and centrifuged (5500 × *g*/10 min/4°C). Following collection of supernatant, which contains cytosolic and mitochondrial fraction, the pellet was vigorously resuspended by syringe/needle in 3 volumes of ice-cold buffer B (20 mmol/l HEPES, pH 7.9; 420 mmol/l NaCl; 0.2 mmol/l EDTA; 1.5 mmol/l $MgCl_2$; 0.5 mmol/l DTT; 0.5 mmol/l PMSF; 25% (v/v) glycerol) and incubated for 30 min on ice. Following centrifugation (12,000 × *g*/20 min/4°C), the supernatant (nuclear extract) was collected. Both extracts were stored in –80°C until further use. Protein content in extracts was determined by the Bradford method (Bradford 1976).

Caspase-3 activity

The PBS-washed cells were lysed in 50 μl of ice-cold lysis buffer (50 mmol/l HEPES, pH 7.4; 0.5% Triton X-100,

a protease inhibitor cocktail tablet, 5 mmol/l DTT; 4°C; 15 min). The lysate was cleared by centrifugation (14,000 × *g*; 4°C; 15 min) and the supernatant total protein determined by the Bradford method (Bradford 1976). The lysate was assayed in 20 mmol/l HEPES, pH 7.1, 2 mmol/l EDTA, 5 mmol/l DTT, containing a protease inhibitor cocktail tablet and caspase-3 fluorescent substrate (Ac-DEVD-AMC; 50 μmol/l) by incubation at 37°C for 60 min. Background fluorescence was subtracted, as obtained in parallel samples with a caspase-3 inhibitor (Ac-DEVD-CHO; 2 μmol/l). The fluorescence was measured at 380/450 nm using an LS 50 B spectrofluorometer (Perkin–Elmer).

Western blot detection of CIDEa

Cells were collected by scraping off in the growth medium to ensure the detached cells are not lost in the rinsing procedure. The cell suspension was centrifuged and the pellet was resuspended in 50 μl of the lysis buffer. Following addition of SDS-PAGE sample buffer (50 μl) it was boiled for 5 min at 95°C. 20–30 μg of total protein/lane was run on 12% SDS-PAGE, blotted onto PVDF membrane and the CIDEa antigen was detected using primary antibody developed in rabbit (ProSci). Horseradish peroxidase-conjugated secondary anti-rabbit antibody and Luminol reagent (Santa Cruz Biotechnology) were used for visualization.

In situ detection of apoptosis (TUNEL)

Detection of apoptosis by TUNEL assay was performed as per manufacturer instructions (Roche). Samples were evaluated under fluorescent microscope Olympus IX70 (Olympus).

Immunocytochemistry

Cells were fixed by adding ice cold methanol and the whole plate was placed overnight into a –20°C freezer. Upon thawing, cells were rinsed twice with PBS and permeabilized by incubating in 0.2% Triton TX-100/PBS (v/v) for 30 min at room temperature. Blocking was performed by incubation for 15 min at room temperature in 3% BSA/PBS (w/v) and was followed by incubation with anti-CIDEa primary antibody (ProSci) diluted 1 : 100 in 3% BSA/PBS (w/v) for 45 min at 37°C. After rinsing three times in PBS, cells were incubated for 30 min at room temperature in fluorescein-conjugated secondary anti-rabbit antibody (Molecular Probes) suspended in 3% BSA/PBS (w/v). Following three washes in PBS, the slides were mounted using fluorescent mounting medium (Dako Cytomation). Confocal microscope FluoView FV1000 (Olympus) was used for evaluation of the slides.

Confocal microscopy

The inverted fluorescent microscope Olympus IX81 with FluoView FV1000 laser scanning unit was employed for single photon microscopy with an Argon laser (457 nm, 488, 515 nm, 30 mW total output) for excitation (Olympus). A pinhole unit (50–800 μm) was used to set confocal conditions.

Cell death assay

Slides for immunocytochemistry were also used for identifying apoptotic cells. Approximately 300 cells total and 100 CIDEa positive cells were counted in random fields of each slide under fluorescent/phase contrast microscope. Apoptotic cells were distinguished based on typical morphological alteration of adherent cells undergoing apoptosis including becoming rounded and condensed. Condensation of cells was also readily apparent after nuclear staining by Hoechst 33258 dye. Cell numbers were then used for estimation of apoptotic cell percentages.

Statistical analysis

Student's *t*-test was used to establish statistically significant differences.

Results

CIDEa overexpression leads to apoptosis in HeLa cells

Previous studies on CIDE proteins stated that overexpression of the protein causes apoptosis (Chen et al. 2000). We have employed T-REx HeLa cells, a cell line stably expressing tetracycline repressor, transfected with pDEST30-CIDEa, which allows regulated and timed CIDEa expression under control of *tet* repressor binding element. First a time course of tetracycline incubation was studied. Time point 0 h clearly shows no expression of CIDEa, indicated that expression of the protein is suppressed in the absence of tetracycline during the 24 h stabilization period post transfection. We found that the maximum expression was reached at 12 h post tetracycline (1 μg/ml) addition, but afterwards expression was declining (Fig 1A). It may imply that overexpression of CIDEa induces apoptosis resulting in cell death, a fact corresponding with previous reports (Chen et al. 2000). The disappearance of CIDEa in the cell population suggests that the transfected cells die, whereas the non-transfected do not. In our case, the majority of transfected cells would perish between 12 and 24 h post-tetracycline addition. Such a short time required for the cells to die may reflect the amount of CIDEa plasmid used for transfection, also shown by others (Inohara et al. 1998), and sensitivity of the cells. While caspase-3 activity varied throughout the time course (data not shown), the percentage

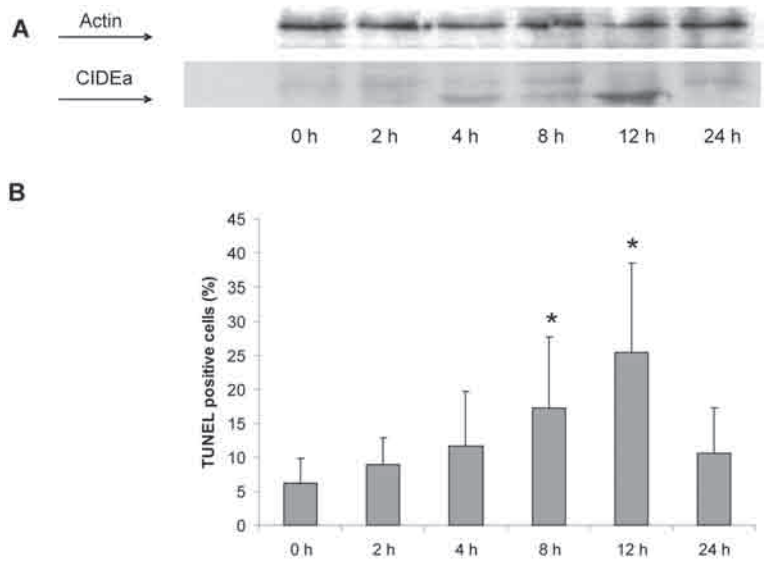


Figure 1. Time-course of CIDEa overexpression and apoptosis in T-Rex HeLa cells. T-REx HeLa cells were transfected with pDEST-CIDEa plasmid and following 24 h stabilization subjected to tetracycline (1 μg/ml) treatment for indicated time periods. **A.** Representative immunoblot of CIDEa presence in samples of transfected HeLa cells treated with tetracycline for 0, 2, 4, 8, 12, and 24 h. Actin served as loading control. **B.** Cells found TUNEL positive were counted and compared with total number of cells. Error bars represent standard deviation determined from three independent experiments. * $p < 0.05$.

of TUNEL positive cells displayed an apparent maximum at 8–12 h with both time points showing significantly higher percentage than time point 0 (Fig. 1B). Hence further experiments conducted used 8 h of tetracycline (1 μg/ml) treatment followed by 2 h in the presence of apoptosis initiators.

Localization of CIDEa in HeLa cells overexpressing the protein

CIDEb was shown to be localized in mitochondria when overexpressed in Chinese hamster ovary cells (Chen et al. 2000) and CIDEa in brown fat mitochondria (Zhou et al. 2003). We also found that CIDEa was localized in mitochondria upon its tetracycline-regulated expression in HeLa cells (data not shown). Immunocytochemistry of CIDEa-overexpressing T-REx HeLa cells shows both nuclear as well as extranuclear localization of CIDEa (Fig. 2). Quantification of CIDEa-positive cells over four independent experiments (100 CIDEa-positive cells counted *per* experiment) yielded higher number of cells displaying either nuclear or mixed, i.e. nuclear as well as extranuclear, CIDEa localization in camptothecin- and valinomycin-treated cells, 50 and 80%, respectively, versus the DMSO-treated cells – 40%.

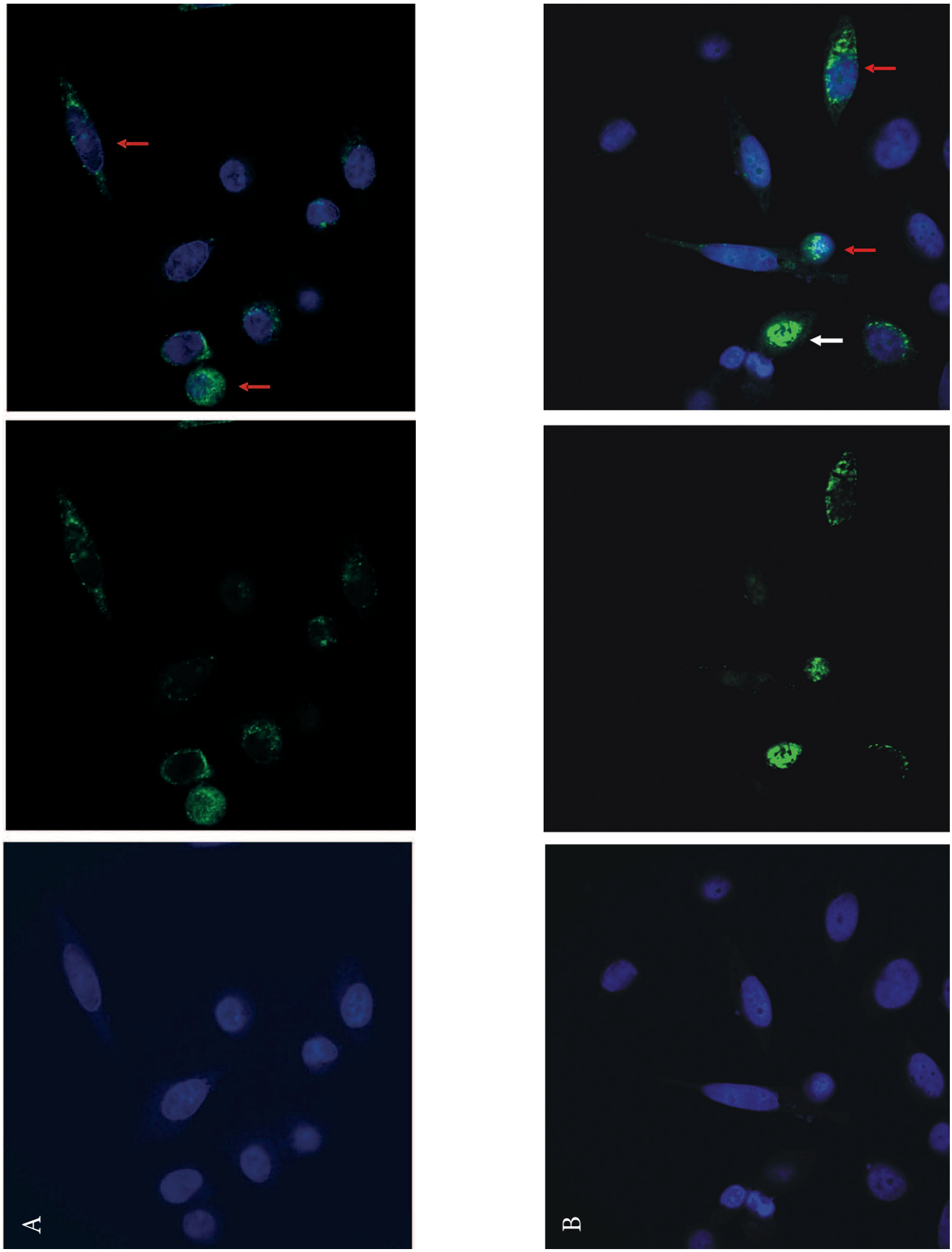
CIDEa redistribution is associated with treatment of HeLa cells with apoptosis initiators

CIDEa expressed under *tet* repressor regulation appears to localize in both extranuclear and nuclear space as shown in confocal microscopy images (Fig. 2). Despite the plausibility of confocal images, we used CIDEa immunodetection in subcellular fractions of CIDEa-overexpressing T-REx HeLa cells to reveal the presence of the protein in general cytosolic, containing mitochondria, as well as nuclear fractions (Fig. 3). Valinomycin effect on CIDEa redistribution is more apparent than in the case of camptothecin and corresponds with the quantification of CIDEa-positive cells displaying at least some nuclear localization of CIDEa (*vide supra*).

Heterogeneous cell population was present in our experiments with variable percentage of cells undergoing apoptosis. Pretreatment of cells with pan caspase inhibitor z-VAD-fmk did not significantly alter the percentage of CIDEa positive cells displaying apoptotic morphology, i.e. cells displaying shrinkage and/or nuclear fragmentation, thus demonstrating the caspase-independence of the process (Fig. 4). On the contrary, the percentage of CIDEa positive/apoptotic cells in samples treated with valinomycin was significantly higher than those treated with DMSO (78 vs. 40%) thus supporting the link between redistribution of CIDEa and apoptosis.

Discussion

Original branding of CIDE proteins as pro-apoptotic factors stems from homology of their N-terminal domain with the corresponding domains of the DFF40/CAD and DFF45/ICAD proteins (Inohara et al. 1998; Lugovskoy et al. 1999).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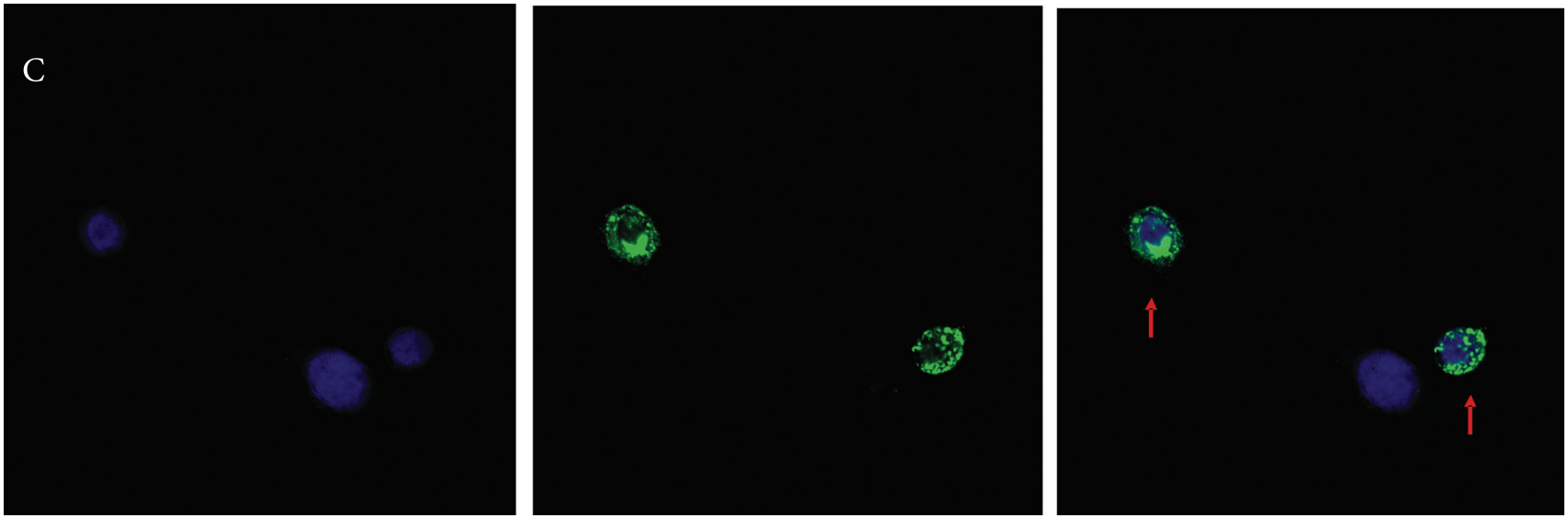

Figure 2. Subcellular localization of CIDEa. T-REx HeLa cells were transfected with pDEST-CIDEa plasmid and following 24 h stabilization subjected to tetracycline (1 μg/ml) treatment for 8 h followed by 2 h of treatment with DMSO (A), camptothecin (2 μmol/l; B) or valinomycin (2 μmol/l; C). Cells were fixed and probed with antiCIDEa antibody followed by AlexaFluor 488-conjugated secondary antibody and Hoechst 33258 dye. Fields shown were visualized under confocal microsope (magnification 400×) using the appropriate wavelengths for AlexaFluor 488 (green fluorescence) and Hoechst (blue fluorescence) dyes, and the two images were merged. White arrows indicate cells showing predominantly nuclear localization of CIDEa, red arrows show cells with nuclear as well as extranuclear localization of CIDEa.

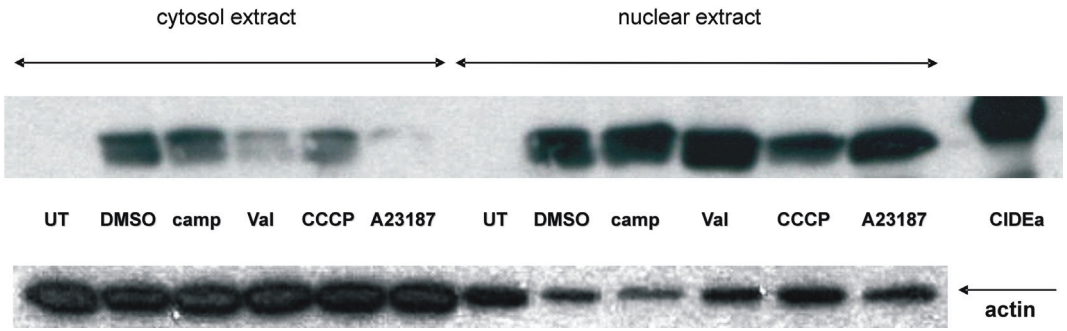

Figure 3. Redistribution of CIDEa from cytosol to the nucleus following apoptosis initiation. T-REx HeLa cells were transfected with empty pDEST or pDEST-CIDEa plasmid and following 24 h stabilization subjected to tetracycline (1 μg/ml) treatment for 8 h followed by 2 h of treatment with the indicated effector. Cells were then collected and cytosolic and nuclear fractions were prepared. 30 μg of total protein from each fraction/lane were separated on SDS-PAGE, blotted onto PVDF membrane and subjected to immunodetection. Primary antiCIDEa antibody was followed by secondary horseradish peroxidase-conjugated antibody to allow chemiluminescent detection. Actin was used as a loading control. UT, empty vector without further treatment; DMSO, pDEST-CIDEa vector treated with DMSO only as vehicle control; camp, 2 μmol/l camptothecin; Val, 2 μmol/l valinomycin; CCCP, 2 μmol/l CCCP; A23187, 2 μmol/l calcium ionophore; CIDEa, recombinant human CIDEa prepared from inclusion bodies.

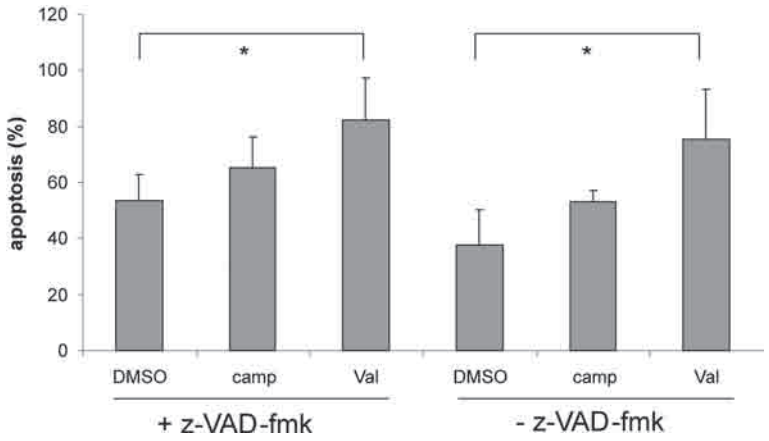

Figure 4. Effect of pan-caspase inhibitor on apoptosis induced by CIDEa overexpression. T-REx HeLa cells were transfected with pDEST-CIDEa plasmid and following 24 h stabilization subjected to tetracycline (1 μg/ml) treatment for 8 h. After brief rinse with PBS, cells were treated for 30 min with 50 μmol/l pan-caspase inhibitor z-VAD-fmk and then further 2 h with the indicated effector. Cells were probed with CIDEa antibody followed by AlexaFluor 488-conjugated secondary antibody and Hoechst 33258 dye. CIDEa positive and apoptotic cells were quantified from random fields, at least 100 cells displaying apoptotic morphology per experiment. Percentage of apoptosis was then calculated as a ratio of CIDEa positive apoptotic cells versus total number of apoptotic cells. Error bars are standard deviations determined from at least three independent experiments. DMSO, cells treated with DMSO only as vehicle control; camp, 2 μmol/l camptothecin; Val, 2 μmol/l valinomycin; * $p < 0.05$.

The final proof that CIDE binds to the homologous domain on DFF45 hence opposing its inhibitory effect on the DFF40 nuclease, which subsequently cleaves DNA, is still missing. Our findings of CIDEa redistribution during incumbent apoptosis, induced either by CIDEa overexpression or in synergy with other apoptotic initiators, make the model, in which the DFF45/DFF40 complex enters the nucleus and CIDEa has to migrate therein to initiate its dissociation, more plausible. This model is also supported by the finding that the whole DFF45/DFF40 complex is imported more readily into the nucleus than its components separated (Neimanis et al. 2007). If CIDEa could disrupt the complex in the cytosol, migration of the free DFF40 nuclease into the nucleus might not be as intense. We found that migration of CIDEa into the nucleus is more apparent during apoptosis initiation by camptothecin and valinomycin and is independent of caspase activity. Hence, in some cell types (Gummesson et al. 2007), CIDEa may constitute another mitochondria-mediated apoptotic pathway alternative to others well-known, e.g. cytochrome c release (Garrido et al. 2006).

Exact pathway leading to CIDEa redistribution is unclear, however, we perceive the different mode of action for each stimulus as the culprit for more pronounced redistribution of CIDEa after valinomycin treatment (Fig. 3). Main target of camptothecin, DNA : topoisomerase I complex, is clearly located in the nucleus. Result of the interaction, i.e. DNA strand breaks, could lead to release of mitochondrial proteins, including CIDEa, only *via* indirect means and after nuclear signaling to mitochondria. On the other hand, valinomycin is a potassium uniporter that causes collapse of mitochondrial membrane potential and its whole transformation into ΔpH (Dlaskova et al. 2008), hence it likely triggers release of mitochondrial proteins as a result of mitochondrial network transformation. Pro-apoptotic proteins Bax and Bak were shown to be involved in normal as well as apoptotic mitochondrial morphogenesis (Karbowski et al. 2006). But we think that role of CIDEa in apoptosis resembles that of cytochrome c rather than that of Bax/Bak.

Previously, the overexpression of CIDEb in COS-1 cells was linked to apoptosis and those studies supported the pro-apoptotic role of the protein (Chen et al. 2000). Our data agree with the pro-apoptotic role of CIDEa, if present in high amounts in HeLa cells, where expression of native CIDEa is absent. In contrast, induction of CIDEa in mouse liver (Viswakarma et al. 2007) or brown fat (Liang et al. 2003; Zhou et al. 2003) was not accompanied by an increase in apoptosis. This can be explained by the existence of a hypothetical co-factor of mitochondrial origin present in certain cell types and capable of preventing CIDEa-dependent apoptosis, suggesting regulation of CIDEa export from mitochondria. A co-factor could be a protein binding CIDEa. Previously, CIDEa was reported to interact with mitochondrial uncoupling protein UCP1 present nearly exclusively in brown adipose tissue, a tissue in which CIDEa did not induce apoptosis (Zhou et al. 2003). Our attempts to verify the proposed interaction between CIDEa and UCP1 using surface plasmon resonance spectroscopy are so far inconclusive (Jezek and co-workers, unpublished data). Similarly, we do not have any evidence that CIDEa may interact with UCP1 homolog, UCP2, despite the pres-

ence of UCP2 in HeLa cells (Valoušková and co-workers, unpublished results). Nevertheless, our data (Fig. 2) support the mitochondrial origin of the postulated co-factor, since overexpressed CIDEa was found to be predominantly localized in mitochondria (Zhou et al. 2003), similarly to its homolog CIDEb (Chen et al. 2000; Liang et al. 2003) or CIDE3 (Liang et al. 2003).

Mitochondrial localization of CIDE proteins may thus serve as sequestering of potentially dangerous proteins much like others previously demonstrated (Kim et al. 2006) that would otherwise put cells into peril of an unavoidable apoptosis. Release of CIDEs from mitochondria and their migration to the nucleus would be then linked to apoptosis. The "resting position" would be represented by CIDE association with the postulated co-factor. Mechanisms of CIDE release from their supposed location within an intermembrane space of mitochondria are unknown, but hitching a ride on small mitochondrial "rafts" as proposed by Skulachev (Skulachev et al. 2004) and hypothesized for ganglioside GD3 (Garofalo et al. 2007) is possible. One can also speculate that CIDE export across the outer mitochondrial membrane may be accomplished by a reverse mode of transport by translocase of outer membrane complexes, by transport through multimers of Bax/Bak or through voltage-dependent anion channel complexes with ceramide, a mechanism either similar or distinct from the ways used by other pro-apoptotic messengers such as cytochrome c, apoptosis-inducing factor, or endonuclease G (Kim et al. 2006). The CIDE transfer into the nucleus should have serious consequences. It would represent important feedback information signaling of e.g. energetic status towards modulation or even execution of apoptosis. As we demonstrated, the CIDE pathway is caspase-independent.

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